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Review

Structure of tau protein and assembly into paired helical filaments

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Abstract

Over the past few years the systematic investigation of paired helical filament assembly from tau protein in vitro has become feasible. We review our current understanding of the structure and conformations of tau protein and how this affects tau's assembly into the pathological paired helical filaments in Alzheimer's disease.

Keywords: Alzheimer's disease; Paired helical filament; Tau protein; Self-assembly; Nucleation

1. Introduction

Alzheimer's disease (AD) is accompanied by a number of structural and metabolic alterations in the brain. Two characteristic hallmarks are the protein aggregates in amyloid plaques (made up mostly of the Aβ peptide, a derivative of the membrane protein APP) and in the neurofibrillary tangles (consisting largely of the microtubule-associated protein tau). Certain forms of AD are related to mutations in the APP gene or the presenilin genes PS1 and PS2 (reviews: [1,2]) so that much of current Alzheimer research is aimed at clarifying the chain of events that lead from the altered gene to the aggregated gene product. Recently in several Alzheimer-related dementias, now summarized as FTDP-17 (frontotemporal dementia with Parkinsonism linked to chromosome 17), mutations in the *tau* gene have been described ([3–7], review: [8]). However, the mechanism for these tau pathologies is still unknown.

In contrast to amyloid plaques the distribution of neurofibrillary deposits correlates well with the clinical progression of the disease [9,10] and thus can be used to subdivide the disease into six stages [9,11]. In this regard, tau deposits have a comparable diagnostic value to the loss of synapses [12,13]. In addition the level of tau in the cerebrospinal fluid becomes elevated in AD which opens up a potential route to early diagnosis [14–16].

Tau is a mainly but not exclusively neuronal microtubule-associated protein (MAP). One of its functions is the stabilization of axonal microtubules [17]; other functions include a role in signal transduction [18,19], interaction with the actin cytoskeleton [20], neurite outgrowth [21–23], interactions with the plasma membrane [24,25], anchoring of enzymes such as protein kinases and phosphatases [26–29], and the regulation of intracellular vesicle transport ([30]; reviewed in [31,32]). Since tau is a highly soluble pro-

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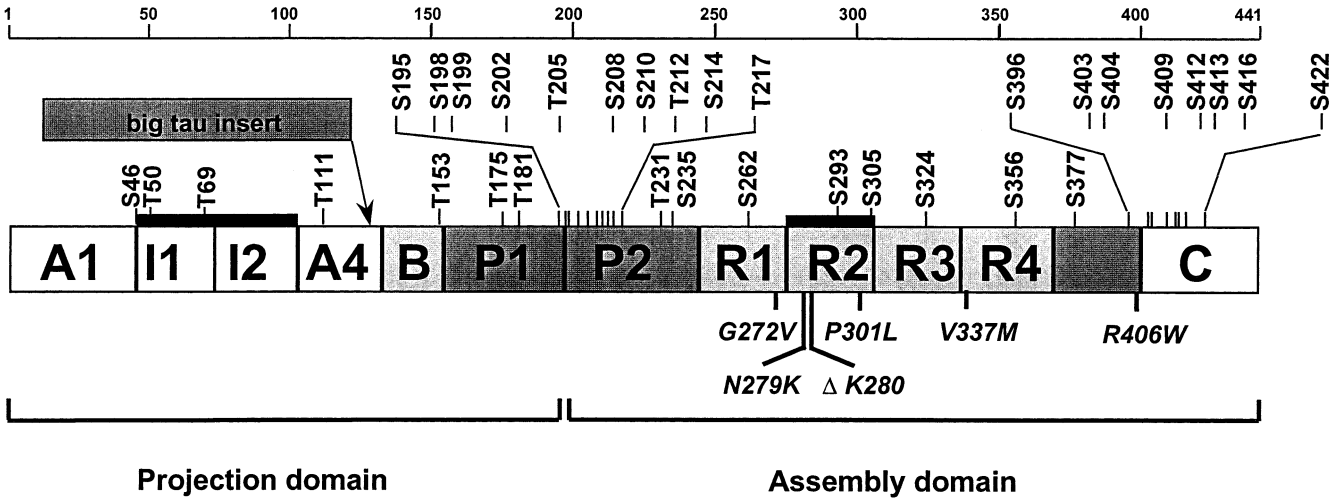

Fig. 1. Bar diagram of tau protein, showing the domains and isoforms generated by alternative splicing. The microtubule binding domain consists of repeats R1–R4 and the flanking regions P (P1, P2, proline-rich, dark shade). Alternatively spliced exons are indicated above (black bars); exon 4a is present in the 'big tau' isoform of peripheral nerves. Exons E2 (insert 1, I1), E3 (insert 2, I2), and E10 (repeat 2, R2) may be absent due to alternative splicing, generating the six isoforms in human CNS. A number of potential phosphorylation sites reported in the literature are listed above the bar, and mutations occurring in FTDP-17 below.

tein, and survives heat, denaturing agents or acid treatment without losing its biological function (the binding to microtubules and the stimulation of their assembly [33,34]), it is counterintuitive that this protein should aggregate into insoluble fibers. Over the past years a number of studies on the structure and assembly of tau, its phosphorylation by various kinases, and its interaction with microtubules have been carried out. Phosphorylation tends to dissociate tau from its natural partner, the microtubule [35–43]. Since this increases the soluble pool of tau it might be an important first step in generating protein for the assembly of paired helical filaments (PHFs). However, the assembly itself appears to depend mainly on other factors (conformation, oxidation, nucleation by other components, see below). In the following we will focus on the question of tau and PHF structure, and the mechanism of filament formation.

2. Structure and conformations of soluble tau

In the human central nervous system the *tau* gene (location on chromosome 17q21) contains 15 exons with the major tau protein isoform being encoded by 11 exons [44]. By alternative splicing of exons 2, 3 and 10 six main isoforms of tau with 352–441 amino acid residues are produced [45–47] (Fig. 1). There are either no, one, or two inserts of 29 residues each near the N-terminus (exons 2 and 3), and three or four homologous stretches of 31 or 32 amino acid residues each, the 'repeats' in the C-terminal half (repeat R2 encoded by exon 10 may be missing). Thus the longest isoform in the CNS has four repeats and two insert 441 residues, the shortest (fetal) isoform 352 residues has three repeats and no inserts. A 'big tau' isoform containing ~300 additional residues (exon 4a) is expressed in peripheral nerves [48,49]. Tau contains either one or two cysteine residues, Cys291 in repeat 2 (present only in four-repeat isoforms and Cys322 in repeat 3 (always present). This difference has an influence on in vitro PHF assembly (see below) and the balance between isoforms may be important within the adult brain. The amino acid composition of tau is dominated by hydrophilic and charged residues, an acidic stretch near the N-terminus followed by mostly basic domains. The repeat region is flanked upstream by a basic proline-rich region (about 25% proline) and downstream by another basic stretch also containing several prolines. Many of these occur in Ser-Pro or Thr-Pro motifs whose phosphorylation is diagnostic of Alzheimer tau. The C-terminal half of tau (repeats plus flanking regions) constitutes the microtubule binding domain [50–52].

Tau has resisted all efforts at crystallization so far (precluding an X-ray crystallographic analysis), and it is too large for a structural analysis by magnetic resonance methods. Therefore, details of the folding of the polypeptide chain are unknown. Most of the available structural data come from electron microscopy, spectroscopy, or small angle X-ray scattering of tau in solution [33,53–57]. Additional information comes from hydrodynamic measurements showing that the polypeptide chain has a highly asymmetric shape (axial ratio of >10 [58]). In summary, these data argue for a natively unfolded conformation of tau with little α-helix and β-sheet [55,56]. This loose, open structure may explain why tau is resistant to heat, denaturants, or acids, because these treatments destroy the compact folding of other proteins but cannot harm tau. These experimental data are corroborated by various secondary structure prediction methods which yield little α and β structure (less than 10% [59]).

Antibodies provide another tool to assess the protein folding if their epitopes are formed from non-contiguous parts of the chain which must come together in space. One example is SMI34, originally raised against phosphorylated neurofilaments [60] which cross-reacts with Alzheimer tau and requires the repeats plus either of the flanking regions in phosphorylated form [61]. Another case is that of the antibodies Alz50 and MC-1 which recognize a conformation of tau typical of AD-tau, are independent of phosphorylation but require regions both near the N-terminus and in the repeats for their epitopes [62,63]. It is remarkable that the three antibodies against 'pathological' tau recognize a folded conformation which brings regions outside the repeats into close vicinity of the repeats, and it is likely that this folding is important for the assembly of tau into PHFs. In this context it is interesting to note that Pin1, a prolyl-peptide isomerase important in mitosis, can interact with tau protein phosphorylated at Thr231 (followed by Pro232). This reverts the conformation that is incompetent to bind to microtubules into a competent one, probably by prolyl-peptide isomerization [64]. Since the proline-rich region of tau contains several Ser/Thr-Pro motifs which are phosphorylated in AD, it will be important to know their influence on the conformation of tau and how such conformational changes are regulated. That tau can indeed adopt distinct conformations has been observed in sequential phosphorylation reactions where the AD-specific phosphoepitope of antibody AT-100 in the proline-rich region can only be generated by a sequential phosphorylation of tau first by GSK-3β (at Thr212) and then by PKA (at Ser214), indicating that pre-phosphorylation at certain sites (e.g. Ser214) can alter the conformation such that other phosphorylation reactions are no longer possible [39].

Dimeric tau derives its importance from the fact that it is a key intermediate in the assembly of PHFs [55,65]. Covalently linked dimers can be formed by disulfide cross-linking, they are therefore not observed in reducing conditions and not expected in healthy nerve cells as long as they maintain their reducing potential. In the electron microscope, tau dimers are seen as rod-like particles (length 25–35 nm, depending on the tau construct [55]), similar to the monomers. Antibody labeling and rotary shadowing electron microscopy suggest that the two monomers of the dimer are arranged in an antiparallel fashion [55]. However, this shape may be oversimplified by the technique used, since in solution the dimers also adopt a mostly random structure like monomers, as judged by circular dichroism spectroscopy [65]. Since three-repeat tau contains only one cysteine residue (Cys322 in R3) only dimers can be formed upon oxidation. The situation is more complicated for four-repeat tau, which bears two cysteine residues (Cys291 in R2 and Cys322 in R3) and can form various dimeric forms and an internal disulfide bridge.

3. Structure of paired helical filaments

3.1. Ultrastructural morphology

The name of PHFs is derived from their electron microscopic appearance as two strands (Fig. 2). They are twisted around one another, such that the cross-over repeats are around 75–80 nm and their apparent width varies between 10 and 22 nm, as if each strand had a diameter of about 10 nm [54]. A fraction of PHFs isolated from Alzheimer brains (about 5%) are not twisted but straight, as if the two protofibrils ran parallel to each other [53]. Other variants of tau fil-

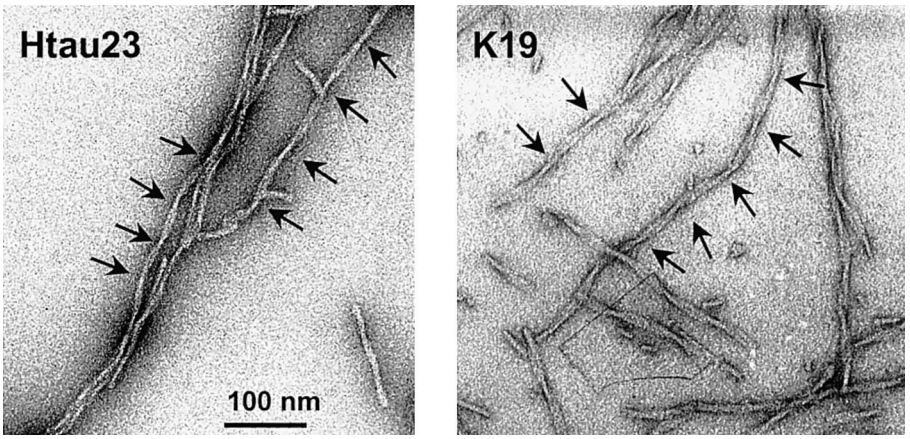

Fig. 2. Electron micrographs of paired helical filaments assembled in vitro. (left) Htau23 (the smallest, fetal isoform) assembled in the presence of the polyanionic cofactor heparin. (right) K19 (a construct comprising repeats R1, R3, and R4) assembled in the presence of poly-Glu. Note the double-stranded twisted appearance with a cross-over repeat of ~80 nm indicated by arrows.

aments are observed in certain familial dementias, showing twisted ribbon-like filaments with an irregular periodicity of 90–130 nm, e.g. in MSDT [66], familial progressive subcortical gliosis [67] or corticobasal degeneration [68]. Image reconstructions suggest that both appearances can be explained by a similar domain structure of the protofibrils [53]. The PHFs usually terminate in an abrupt fashion without fraying out, suggesting that the two strands are not separate entities on a molecular level. This would be explained if the constituent subunits of tau protein were distributed over both subfibers. Images from atomic force scanning microscopy emphasize a ribbon-like structure, twisting with the same cross-over distance of ~80 nm, but without the subdivision into two strands [69]. The main problem in all models of PHFs thus far is that their protein subunits cannot be clearly delineated so that the packing of molecules is still unknown.

3.2. Biochemical composition

It is widely accepted that all six tau isoforms occur in PHFs [70,71] while other tangle proteins are only peripherally associated (e.g. ubiquitin [72]). However, the situation of isoform composition can be different in other dementias with tau pathology where four-repeat isoforms predominate (e.g. FTPP-17 [6,73]) or three-repeat isoforms (e.g. Pick's disease [74]). The tau protein which is found in filaments is hyperphosphorylated [61,75–78]. Important constraints for structural models of tau filaments come from proteolytic cleavage and antibody labeling experiments, suggesting that the structural core of PHFs contains the equivalent of three repeats [79]. The remainder of tau (both N- and C-terminus) forms the 'fuzzy coat' and can be removed by proteases such as pronase [80–82].

A feature of many amyloid deposits is that the protein fibrils are enriched in β-sheet content [83]. The β strands usually run perpendicular to the helix axis forming a 'cross-β' motif [57,84]. Such fibers can be stained with certain dyes such as Congo red or thioflavine S which are thought to interact with the repeating β strands [85]. Since PHFs react with these dyes to some extent (see below) it had been assumed that PHFs had a cross-β structure of subunits. Repeated β strands should reveal an axial periodicity of about 0.47 nm by X-ray diffraction which was reported in one case [57] but could not confirmed in other studies [56]. Similarly they should generate a maximum around 1620–1630 cm^{-1} in a Fourier transform infrared (FTIR) spectrum, in contrast to the observed maximum at 1658 cm^{-1} which is typical of a low content of β structure [65]. Thus the data obtained from X-ray diffraction for PHFs could not be confirmed by FTIR spectroscopy [56]. An explanation of these inconsistencies could be the difficulty of preparing PHFs from brain tissue without any impurities and the high sensitivity of X-ray diffraction to any regular structure. However, the results from X-ray diffraction and FTIR spectroscopy do

not indicate an extended β-sheet structure. These results are consistent with the near-absence of secondary structure with full-length tau in solution noted above (but do not exclude the possibility of a local β structure in PHFs which would be below detectability by spectroscopic methods, see final Note).

4. Assembly of tau into PHFs in vitro

4.1. The repeat region of tau is necessary and sufficient for PHF assembly

Tau aggregates have been observed in the brains of patients affected with AD and related tauopathies, but so far no tau filaments have been observed in any model systems such as cells transfected with tau or transgenic mice. Therefore our knowledge about the mechanism of tau assembly into PHFs stems largely from in vitro data. To understand the principles it is necessary to assemble the subunits into the fibers in vitro and study the structure both in the subunit and in the polymeric states. For PHFs the progress has been slow, primarily because tau is soluble in most circumstances. Many peptides have a tendency to aggregate in certain conditions, but the significance remains unclear if the aggregates do not resemble the native fiber (for examples see [86]). Thus, tau isolated from brain tissue can form fibers of homogeneous diameter [87,88], but the relationship to PHFs remains unclear. Tau can also form filaments in some reducing conditions and in the presence of free fatty acids, but these do not show the typical paired helical filament appearance [89,90].

Bona fide PHFs, showing the appropriate diameter and periodicity, were first assembled from recombinant tau constructs containing essentially the repeats [55], albeit at low efficiency. Assembly was strongly promoted by the covalent dimerization of tau via Cys322, whereas site-directed mutagenesis of Cys322 to alanine inhibited PHF assembly [65]. Three-repeat tau constructs, having one cysteine, can be dimerized by oxidation and form PHFs readily, while four-repeat constructs tend to form intramolecular cross-bridges and do not readily dimerize and assemble. Nevertheless, even with dimerized three-repeat constructs the assembly was slow and inefficient. Moreover, the difficulty remained that full-length tau would not assemble, and that native PHFs contained both three-repeat and four-repeat isoforms.

4.2. Polyanions stimulate PHF formation in vitro

A further important step was the observation that several polyanionic cofactors greatly facilitate the formation of PHFs from tau protein in vitro. These polyanions can be heparin or other sulfated glycosaminoglycans [91,92], RNA [93] or polyglutamic acid [92,94]. A role of these substances in PHF formation is supported by the finding that both sulfated glycosaminoglycans [95–97] and RNA [98,99] are found to be associated with neurofibrillary tangles. These polyanionic cofactors stimulate the assembly of full-length tau, both with three and with four repeats, within a few days. A systematic variation of the domain composition showed that all tau proteins would assemble into PHFs provided that they contain at least two repeats [93]. This emphasizes the role of the repeat domain of tau in PHF assembly, consistent with their presence in the cores of Alzheimer PHFs. Secondly, the assembly still required disulfide cross-linking and could be prevented by reducing agents such as dithiothreitol. Aggregation experiments with small peptides in the presence of polyanions (heparin or polyglutamic acid) comprising only parts of the repeat region (the 18-amino acid residue conserved part of the repeats) revealed that only peptides from the third repeat and to a lesser extent from the second repeat formed fibers, which, however, are much thinner and therefore do not resemble PHFs [92,100]. An analysis of the influence of other regions of the repeat on PHF formation awaits investigation.

In short, assembly of tau protein in oxidizing conditions and in the presence of cytosolic nucleating agents fulfills all the requirements for bona fide PHF formation (full-length molecules, all isoforms). This has opened the way for detailed studies of the kinetic properties of PHF assembly, for analyzing PHF assembly in cell models, and for studying the structure of PHFs.

4.3. PHF formation is a nucleation-dependent process

The development of a quantitative assay for PHF

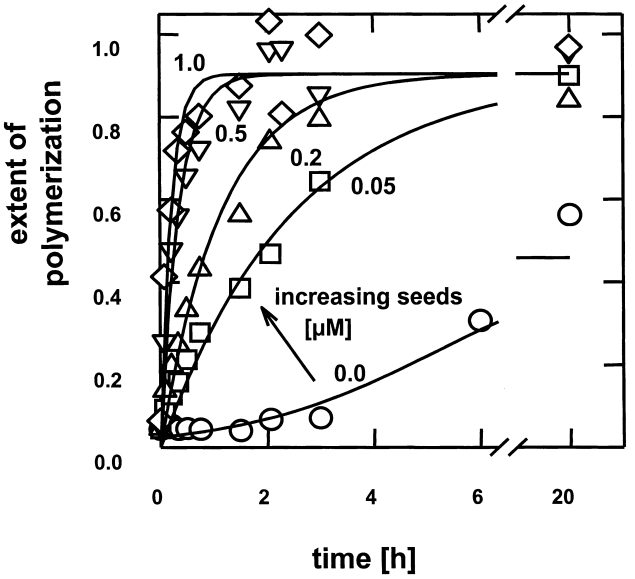

Fig. 3. Fluorescence assay of PHF assembly illustrating the effect of nucleation. Tau construct K19 was assembled in the presence of poly-Glu and in the presence or absence of assembly seeds obtained from pre-assembled PHFs and the extent of polymerization was measured by ThS [94]. Bottom curve (circles): no seeds (spontaneous nucleation, note the lag phase). Upper curves: increasing concentration of exogenous seeds (seeded assembly, no lag phase). Adapted from [102].

formation using thioflavine S [94] which is known to stain not only amyloid but also PHFs [65,80,101] opened the way to a more detailed study of the mechanism of PHF formation (Fig. 3). Using this assay (with correlated electron microscopy to monitor the morphology of the filaments), assembly studies revealed that even after formation of dimers and in the presence of polyanions a kinetic barrier existed, which prevented the rapid formation of filaments [91,94]. This suggested that a nucleation step beyond dimerization is rate limiting. The slow nucleation step, which requires an estimated number of four to seven subunits (=tau dimers) to form the nucleus and therefore requires high concentration of tau, could be circumvented and filament formation drastically accelerated (to the range of minutes to hours, depending of the tau construct used) by adding small amounts of seeds [102]. These seeds can be made either from authentic PHFs derived ex vivo (isolated from the brains of AD patients) or from filaments made in vitro from various tau constructs. Under such conditions even tau protein without a cysteine residue (namely Cys322Ala), which can only inefficiently dimerize non-covalently, is now able to form filaments in vitro, albeit at a strongly reduced rate compared to the covalently cross-linked variant. The need for stoichiometric amounts of polyanions to achieve quantitative filament formation [91,92,94] is also observed under seeding conditions. Thus even in the presence of seeds dimeric tau will not readily assemble into filaments without polyanions, which are therefore also needed for elongation [102] (Fig. 4).

These features make PHF assembly broadly similar to amyloid fiber aggregation from Aβ in AD [83,103], to Lewy body aggregation from α-synuclein in Parkinson's disease [104], to the aggregation of huntingtin in Huntington's disease [105] or to prion protein aggregation in prion diseases [106]. Whether heterologous nucleation (observed in amyloid formation) is also possible for tau is not known presently. In summary, the best conditions for PHF formation in vitro are (1) oxidative conditions to allow tau dimerization, (2) presence of polyanions and (3) small amounts of seeds.

4.4. PHF formation is retarded by phosphorylation

Since tau appears in the hyperphosphorylated form in PHFs [75], it is commonly assumed that this hyperphosphorylation of tau causes its detachment from microtubules and promotes its assembly into PHFs. However, in the absence of polyanions full-length tau is not able to form filaments, and in the presence of polyanions phosphorylated tau does not aggregate faster than unmodified tau [107]. In a systematic study using different kinases (MARK, PKA, MAPK, GSK3) it was shown that phosphorylation of tau by proline-directed kinases (MAPK and GSK3, known to phosphorylate tau in the regions adjacent to the repeat domain) had a weak negative effect on tau–microtubule interactions as well as on PHF assembly [108]. By contrast, phosphorylation of tau by MARK and PKA (at the KXGS motifs within the repeat region, especially Ser262, and at Ser214 in the flanking region) strongly inhibits tau's attachment to microtubules. Significantly, this type of phosphorylation tends to inhibit PHF formation, contrary to earlier expectations. Therefore phosphorylation tends to protect tau against PHF formation, rather than promoting it.

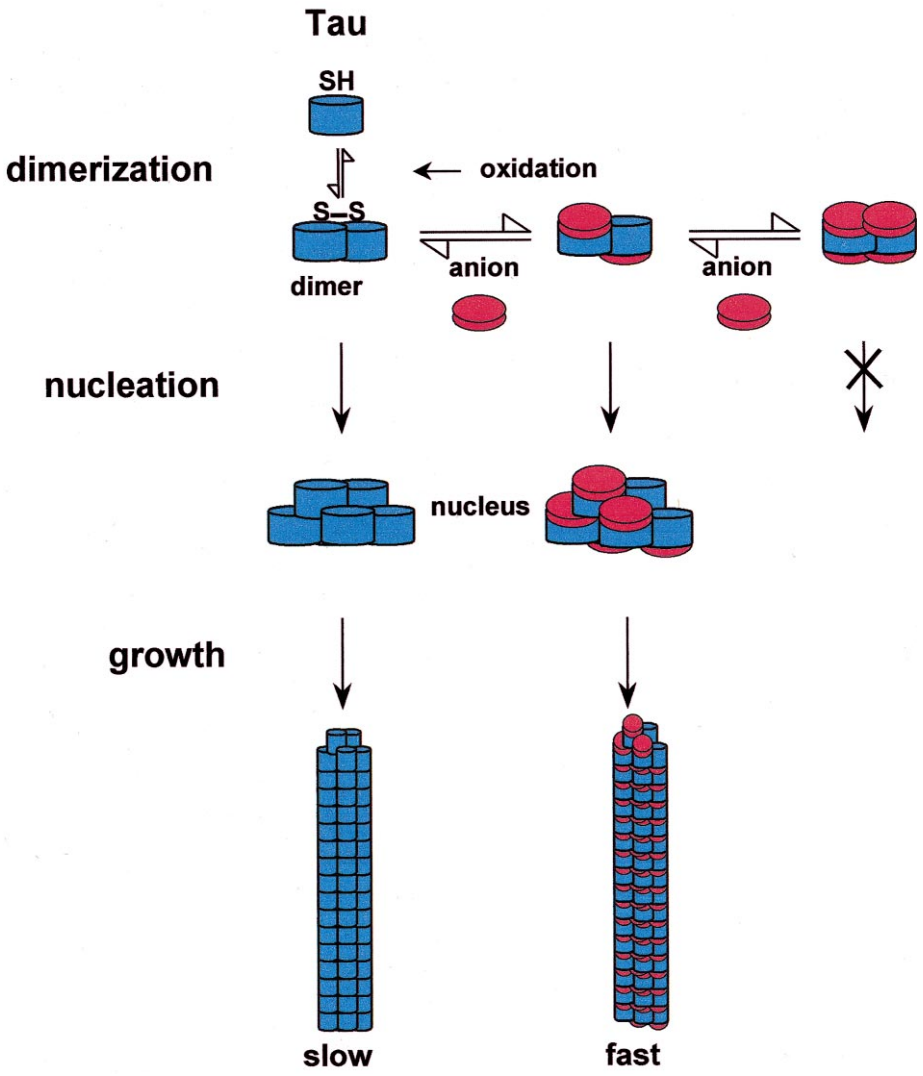

Fig. 4. Diagram of tau dimerization, PHF nucleation and assembly. Tau monomers dimerize either covalently by oxidation (disulfide bridge formation, stable dimer) or non-covalently (unstable dimer). The dimer is the effective building block of PHFs. Nucleation is slow in the absence of polyanions but strongly accelerated in their presence (e.g. RNA, poly-Glu, heparin). Elongation (with or without exogenous seeds) is slow in the absence of polyanions or by prevention of dimerization, indicating that a dimer–polyanion complex is incorporated into PHFs. Excess of polyanion is inhibitory. Note that the stoichiometry and the arrangements are not known and are only shown for illustration. Adapted from [102].

4.5. PHF formation and tau gene mutations

The finding of mutations in the *tau* gene that correlate with the development of disease with tau deposits raises the question of what role the mutations play during tau filament formation. Recent studies with recombinant wild-type or mutant proteins as found in disease (e.g. FTDP-17) suggest that some but not all types of tau mutations lead to an accelerated filament formation [100,109,110]. Therefore the reason for disease development might in some cases be directly related to the filament formation propensity of the proteins while in other cases an altered function in tau–microtubule interaction might be involved [73].

4.6. PHF formation and animal models

Using animal models (e.g. transgenic mice expressing mutant APP or presenilins) amyloid deposition is

observed while there is no evidence so far for neurofibrillary pathology. Since subtle sequence changes even in introns in the *tau* gene can influence the function and result in disease (see FTDP-17) one could also speculate that other variations could prevent tau pathology. Using three- or four-repeat recombinant mouse tau it could be shown that at least at the protein level there are no reasons why neurofibrillary pathology is not observed in transgenic mice. In vitro all mouse tau proteins could be aggregated in the presence of polyanions in a way indistinguishable from human tau [111]. Therefore the absence of tau pathology in transgenic mice must be explained by other yet unknown factors. Simple overexpression of tau in transgenic animals also failed to reveal neurofibrillary pathology although staining with certain disease-specific antibodies has been reported (e.g. 12E8 for phosphorylated Ser262, AT-8 for Ser202/T205 [112–114]).

5. Future directions

A crucial question remains – how does the assembly pathway outlined above pertain to neurons in Alzheimer brain tissue? Cells normally have a reducing environment maintained by an excess of glutathione. This ensures the successful scavenging of reactive oxygen species and free radicals, and it depends on a well-functioning energy metabolism. AD appears early in large pyramidal neurons of the hippocampus which have a high metabolic rate and thus might be expected to be most vulnerable to toxic effects (e.g. glutamate excitotoxicity, toxic Aβ, radicals generated by activated microglia, etc.). In addition, mitochondrial DNA lacks the repair system of nuclear DNA so that oxidative phosphorylation becomes less efficient with age, as seen from experiments involving the mitochondria from Alzheimer tissue [115]. The relative longevity of man may explain why other animals, including aged sheep or monkeys [116] or transgenic mice overexpressing the amyloid precursor protein [117] or human tau [112], do not show neurofibrillar pathology comparable to that of AD.

Tau pathology is characteristic not only of AD but also of several other neurodegenerative disorders. Tau appears in aggregated filaments of varying morphology, with different isoform composition and a hyperphosphorylated form. The identification of the *tau* gene as the genetic lesion in several hereditary forms of dementias lends credence to the view that tau filaments might be sufficient to cause nerve cell degeneration. Recent advances in experimental in vitro systems to study the aggregation of tau protein into filaments have opened the way towards systematic studies of the mechanism of filament formation as well as the development of inhibitors of the process. Although much progress towards the mechanism of tau pathology has been made, several questions remain open and have to be addressed in the future. (1) How do the parameters influencing tau filament formation in vitro relate to the disease process (i.e. oxidation, truncation, anionic cofactors)? (2) What is the role of hyperphosphorylation and other modifications of tau during filament formation? (3) What is the first nucleation step in the formation of filaments in vivo? (4) Last but not least, there is a need for an in vivo system (cell culture of animal model) for tau pathology.

6. Note added in proof

We have recently found that a short hexapeptide stretch at the beginning of the third repeat (306-VQIVYK-311) that induces PHF aggregation by forming beta structure [118].

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